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INTRODUCTION

The reactive stroma microenvironment is important to prostate cancer, however specific mechanisms of its role in regulating prostate cancer progression are poorly understood. Human lung, breast, and colon carcinomas all exhibit alterations in the stromal compartment, yet the specific composition, the origin of reactive cells, and the factors that regulate this evolution are understudied. The myofibroblast appears to be a key component of carcinoma associated reactive stroma. This highly synthetic cell with a contractile phenotype is not observed in the normal human prostate and its appearance positively correlates with prostate cancer severity. Recent studies have suggested that myofibroblasts at sites of reactive stroma might originate from circulating "fibrocytes" linked to the hematopoietic lineage. CD34, a single chain transmembrane glycoprotein is important in homing and adhesion of hematopoietic progenitor cells. This cell surface marker is expressed by fibrocytes and is emerging as an important player in the normal wound repair response. Full thickness skin wounds showed robust recruitment of CD34+ to the dermis, with subsequent differentiation to myofibroblasts. Similarly, mouse lungs treated with allergen resulted in recruitment of circulating CD34+ progenitors to bronchial tissue where they subsequently differentiated to myofibroblasts. This study also showed allergic asthma patients had fibrocytes in bronchial mucosa that were positive for CD34, collagen I, and smooth muscle alpha actin, suggesting that progenitors were from circulating bone marrow derived cells. It has also been demonstrated that CD34+ fibrocytes grown in culture could be induced to myofibroblast differentiation *in vitro* by TGF- β . Finally, an additional cell surface marker, CD14, seems to be emerging as a more specific markers of fibrocytes. While these studies have demonstrated an involvement of fibrocytes in re-establishing tissue homeostasis, the precise origin of these progenitor cells and their role in reactive stroma is not clear. It is our hypothesis that human prostate cancer reactive stroma is composed of myofibroblasts recruited from a circulating population of CD14/CD34+ fibrocytes.

BODY

Task 1: To determine whether marrow-derived circulating human fibrocytes can be recruited to sites of reactive stroma and if they are the progenitor for myofibroblasts in the tumor microenvironment of human prostate cancer.

Progress:

1) Protocols for whole marrow transplantation experiments have been devised and optimized. Two colonies of reporter mice were expanded and used as donor mice in these transplantation studies: (1) mice transgenic for beta-actin promoter driving cyan fluorescent protein (CFP) and (2) mice transgenic for smooth muscle alpha actin promoter driving LacZ. Athymic *nu/nu* mice obtained from Charles Rivers were used as recipient mice in these irradiation studies. Briefly, bone marrow was derived from each set of reporter mice and purified. Athymic mice of approximately 8 weeks of age were irradiated with a split dose of 10.5 Gy and injected via tail vein with approximately 1×10^6 whole marrow cells. Engraftment of marrow was assessed via FACS at 4 and 8 weeks, with engraftment approaching 80% by 8 weeks. A total of 75 athymic mice have been successfully transplanted (32 with CFP positive marrow and 43 with p2600Int/LacZ positive marrow). Two-way xenografts containing LNCaP human prostate cancer and Matrigel were implanted subcutaneously in the flank region of a subset of the transplanted mice and were grown for 7, 14, 21, and 28 days. Two independent injections have been carried out at each timepoint for both sets of transplanted mice, with bilateral injections sites for each mouse. All xenograft tumors have been harvested and embedded, for a total of 64 tumors for each group of mice. Immunohistochemistry for CFP, in conjunction with other markers of reactive stroma, will allow for true quantitation of bone marrow recruitment to these tumors. SMA expression through expression of LacZ would indicate phenotypic switch to a myofibroblast.

2) Adoptive transfer experiments were performed to assay for the recruitment of human CD34+ cells to 2-way xenografts containing human LNCaP prostate cancer cells and matrigel. 1×10^6 human sorted CD34+ cells from peripheral blood were injected into an athymic mouse containing a 2-way xenograft and grown for 7 days. Preliminary data shows some recruitment of cells based on CD34+ immunohistochemistry and morphology of cells that have been recruited to the site. Further analysis of these recruited cells is pending. Parallel experiments in which CD34+ cells were combined with LNCaP and matrigel were performed. Differential survivability of LNCaP cells was observed while CD34+ cells seemed to take residence in clusters.

3) Pilot studies have been performed with adoptive transfer of CD14+ monocytes into athymic mice containing LNCaP xenografts. Tumors have been harvested and embedded in paraffin blocks for analysis.

4) Acquisition and banking of human biopsy samples from several human carcinoma tissues to characterize the expression of various stromal markers in an effort to further understand the microenvironment across distinct tissue groups.

Immunohistochemistry has been performed for a battery of markers on prostate, lung, colon, pancreas, thyroid, breast, and brain tissues. Preliminary observations demonstrate a reactive stroma rich in vimentin and CD34. These stromal changes appear to be heterogeneous between various cancer patients. Images are being acquired and analyzed using multispectral deconvolution microscopy (Nuance, CRI). Quantitation of heterogeneity between normal, PIN, and cancer specimens will be performed on prostate arrays.

5) Co-culture studies have been initiated that will combine human CD14+ cells with LNCaP cells overexpressing a constitutively active form of TGF- β in order to address the role of TGF- β in reactive stroma induction. A protocol has been developed to effectively generate LNCaP spheroids in culture to be combined with CD14+ monocytes. Initial culture conditions are still being worked out, in order to maximize survivability of both cell populations.

KEY RESEARCH ACCOMPLISHMENTS

- A total of 75 athymic mice have been successfully transplanted (32 with CFP positive marrow and 43 with p2600Int/LacZ positive marrow). These animals will serve as reagents for many of the experiments proposed in this DOD grant.
- Two-way xenografts containing LNCaP human prostate cancer and Matrigel were implanted subcutaneously in the flank region of a subset of the transplanted mice and were grown for 7, 14, 21, and 28 days.
- Two independent injections have been carried out at each timepoint for both sets of transplanted mice, with bilateral injections sites for each mouse. All xenograft tumors have been harvested and embedded, for a total of 64 tumors for each group of mice.
- Preliminary data in adoptive transfer studies shows some recruitment of cells based on CD34+ immunohistochemistry and morphology of cells that have been recruited to the site. Adoptive transfer studies using human CD14+ monocytes have also been performed. These are proof-of-principle studies that demonstrate tremendous potential in creating a model to study human cell recruitment in a mouse host.
- Human biopsy specimens from prostate, lung, colon, pancreas, thyroid, breast, and brain tissues containing regions of normal and cancerous tissue have been banked. Several patients are in each tissue cohort. All tissues were dual stained with a combination of any two of the following markers: CD34, CD14, vimentin, tenascin, CD31, CD11, CD68, and smooth muscle alpha actin. The tissues are currently being imaged and analyzed.
- Human LNCaP cells expressing a constitutively active form of TGF-beta or empty vector have been successfully grown in spheroids and maintained for weeks in culture. These spheroids will serve as the backbone for co-culture studies to examine the fate of CD14+ cells exposed to a high level of TGF-beta in a cancer niche.

REPORTABLE OUTCOMES

- 2008 Department of Molecular and Cellular Biology Symposium, Baylor College of Medicine—Abstract and poster presentation
- 2008 Medical Scientist Training Program, Baylor College of Medicine—Abstract and poster presentation
- 2008 Dan L. Duncan Cancer Center, Baylor College of Medicine—Oral presentation
- 2008 Keystone Symposium on Cancer, Stem Cells, and Aging, Singapore Biopolis—Abstract and poster presentation

CONCLUSION

All work discussed in this report is preliminary, and thus no conclusions can be drawn until further work is completed. At this time, much of the data does point towards a circulating progenitor of reactive stroma, that seems to be quite important in giving rise to much of the heterogeneity seen in prostate cancer. Furthermore, this circulating progenitor may be immune in nature, displaying properties of a monocyte as it arrives at the site of cancer and differentiating into a myofibroblast or macrophage, depending on the signals received in the tumor microenvironment.

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- 3) Abe, R., Donnelly, S. C., Peng, T., Bucala, R., and Metz, C. N. Peripheral blood fibrocytes: differentiation pathway and migration to wound sites. *J Immunol*, 166: 7556-7562, 2001.
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- 5) Pilling, D., Tucker, N. M., and Gomer, R. H. Aggregated IgG inhibits the differentiation of human fibrocytes. *J. of Leukoc Biol.*, 79(6):1242-51, 2006.

APPENDICES

1. Curriculum Vitae

David Barron

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EDUCATION

M.D. Baylor College of Medicine, (anticipated) 2011
Houston, TX

Ph.D. Baylor College of Medicine, (anticipated) 2010
Houston, TX
Molecular and Cell Biology
Advisor: David Rowley, Ph.D.

B.S. Rice University, 2003
Houston, TX

Biochemistry; Biology
Study Overseas The University of Oxford, 2002
Oxford, England
Biological Chemistry and Molecular Genetics

HONORS and AWARDS

1999 Rice University Herbert Allen Scholarship
2001-2002 Baylor College of Medicine's Student Medical and Research Training
(SMART) Program
2002 Inducted into Alpha Alpha Chapter of Phi Lambda Upsilon Honors
Chemistry Society
2002 University of Oxford Biochemistry Society
2002 Rhodes Scholar nominee for Rice University
2003 Who's Who Among American College Students
2003 Graduated cum laude in Rice University Departments of Biochemistry
and Cell Biology
2008 Co-Chair of Planning Committee for Molecular and Cellular Biology
Departmental Symposium
2008 Scholarship for Keystone Symposium on Stem Cells, Cancer, and Aging
in Singapore

RESEARCH EXPERIENCE

2001 Summer	<p>Baylor College of Medicine, Department of Pulmonary and Critical Care Medicine, Aladin Boriek, Ph.D.</p> <p>Completed the initial stages on a research project involving the analysis of rib cage and diaphragm coupling in a canine model.</p>
2001 Fall	<p>Baylor College of Medicine, Department of Pulmonary and Critical Care Medicine, Aladin Boriek, Ph.D.</p> <p>Completed research on the study of signal transduction in mice myocytes.</p>
2002 Spring	<p>University of Oxford, Department of Biochemistry, Professor Edwin Southern</p> <p>Assisted in devising specific antisense oligonucleotides to effectively silence c-Raf, a transcription factor partly responsible for the progression of cancer.</p>
2002 Summer	<p>Baylor College of Medicine, Department of Pulmonary and Critical Care Medicine, Aladin Boriek, Ph.D.</p> <p>Conducted osmotic stress experiments on connective tissue cells and subsequently tested for the activity of the transcription factor NF-κB.</p>
2002 Fall	<p>Baylor College of Medicine, Department of Pulmonary and Critical Care Medicine, Aladin Boriek, Ph.D.</p> <p>To further understand the molecular mechanisms of asthma, exposed mouse lung tissue to mechanical stress and subsequently tested for the activity of NF-κB.</p>
2005 Fall-Present	<p>Baylor College of Medicine, Department of Molecular and Cell Biology, David Rowley, Ph.D.</p> <p>Currently investigating the putative origins of reactive stroma myofibroblasts and their mechanisms of recruitment to sites of early prostate cancer.</p>

PUBLICATIONS

Barron D, Kumar A, and Boriek M. The role of mechanical stress in skeletal myocytes: MAPK signal transduction pathways. Reviews in Undergraduate Research, Vol. 1, 8-20, 2002.

Kumar A, Lnu S, **Barron D**, Moore J, Corry D, Schwarz R, and Boriek M. Mechanical stretch activates nuclear factor-kappaB, activator protein-1, and mitogen activated protein kinases in lung parenchyma: Implications in asthma. FASEB J. 2003 Oct;17(13):1800-11.

PRESENTATIONS

- | | |
|------|--|
| 2001 | Biomechanics of respiratory coupling in a canine diaphragm model.
Baylor College of Medicine Student Medical and Research Training (SMART) Program |
| 2002 | The Role of NF-kB as an Upstream Regulator of Signal Transduction Pathways
Baylor College of Medicine Student Medical and Research Training (SMART) Program |
| 2007 | The Origin of Reactive Stroma in Prostate Cancer
5 th Annual Dan L. Duncan Cancer Center Symposium |
| 2008 | Further Defining Reactive Stroma in Prostate Cancer
Dan L. Duncan Cancer Center Prostate Program Seminar Series |

TEACHING EXPERIENCE

- | | |
|-----------|--|
| 2000-2001 | Teaching Assistant, General Physics |
| 2001 | Teaching Assistant, General Chemistry |
| 2001 | Teaching Assistant, Biology |
| 2001 | Tutor, Organic Chemistry, General Chemistry, and Biology |
| 2002 | Tutor, Biochemistry and Cell Biology |
| 2002 | Teaching Assistant, Biochemistry |
| 2002-2003 | Tutor, Organic Chemistry |
| 2006 | Teaching Assistant, Cell Biology and Histology |
| 2007 | Mentor, Baylor College of Medicine SMART Program |
| 2008 | Mentor, Baylor College of Medicine SMART Program |
| 2008 | Teaching Assistant, Cell Biology and Histology |

GRANTS and FELLOWSHIPS

- | | |
|----------------|--|
| 2001
Summer | Baylor College of Medicine SMART program grant |
|----------------|--|

2002 Summer	Baylor College of Medicine SMART program grant
2003-2005	Ruth L. Kirschstein National Research Service Award
2005	BP America Biomedical Scholars Program
2008	Department of Defense Prostate Cancer Predoctoral Training Award

EXTRACURRICULAR ACTIVITIES

American Medical Student Association (AMSA)—Baylor College of Medicine Chapter
 American Medical Association (AMA)—Baylor College of Medicine Chapter
 Member of the MSTP Student Operating Committee (SOC): 2004-2006
 Member of Student Admissions committee for Baylor College of Medicine: 2005-present
 Mentor for Baylor College of Medicine Peer Resource Network (PRN): 2004-2005

PROFESSIONAL ORGANIZATIONS

Affiliates of the American Chemical Society
 American Association for the Advancement of Science

2007-2008 Summary

- 2008 Scholarship for Keystone Symposium on Stem Cells, Cancer, and Aging in Singapore**
- 2008 Mentor, Baylor College of Medicine SMART Program**
- 2008 Further Defining Reactive Stroma in Prostate Cancer
Dan L. Duncan Cancer Center Prostate Program Seminar Series**
- 2008 Department of Defense Prostate Cancer Predoctoral Training Award**
-

2. Abstract

a. Keystone Conference on Cancer, Stem Cells, and Aging.

Quiescent fibroblasts and smooth muscle make up the mesenchymally-derived component of normal human stroma. These cells maintain tissue homeostasis and respond to disruptions in the tissue microenvironment by forming a reactive stroma phenotype. The principal component of prostate cancer reactive stroma is the myofibroblast (also known as carcinoma-associated fibroblasts, CAFs), a highly synthetic and contractile cell type. Myofibroblasts also make up reactive stroma associated with mammary, lung, colon, and stomach carcinoma, suggesting that they play a general role in a reactive stroma host response to carcinoma. Our previous studies have shown that reactive stroma is tumor-promoting in prostate cancer models. The origin and mechanism of myofibroblast / CAF recruitment is unknown. Accordingly, understanding the origin and biology of this recruitment is important since novel approaches that target these cells may have therapeutic value. Recent studies suggest that myofibroblasts at sites of wounding may originate from the bone marrow CD34 positive progenitor cells and our preliminary data shows that foci of reactive stroma in human prostate cancer are CD34+. Moreover, our data shows that some acini lining stromal cells in the normal prostate gland are CD34+.

It is our hypothesis that prostate cancer reactive stroma is composed of myofibroblasts / CAFs recruited from either tissue-fixed or circulating CD34+ fibrocytes. As a modification of the differential reactive stroma (DRS) xenograft model system we have reported, we have also developed a "matrix trapping" procedure to address host cell recruitment to matrix implants. Cells recruited to matrix traps stained negative for smooth muscle α -actin and positive for tenascin, pro collagen type 1, and CD34. Preliminary data from tail vein injections of human CD34+ cells into nude mice containing LNCaP xenografts shows that these cells can be recruited. These data suggest that reactive stroma may originate from circulating, marrow-derived progenitors. Bone marrow transplant studies have been initiated using both CFP and smooth muscle α -actin reporter mice as marrow donors to further address the origin of reactive stroma cells and their potential for differentiation to myofibroblasts / carcinoma-associated fibroblasts.

SUPPORTING DATA

1. Development of the Co-Culture Model.

We have developed and assessed the feasibility of the co-culture model in defined media. LNCaP cells were first engineered to express constitutively active HA-TGF- β 1 or empty vector control via retroviral transduction (bicistronic expression of HA-TGF- β 1 and EGFP marker). Expression of biologically active HA-TGF- β 1 was verified and LNCaP cells (1×10^6) were seeded onto soft agar (1%) in 100 mm plates and allowed to develop into spheroids on the surface of the agar for 3-4 days. This produces spheroids between 100-250 mm in diameter that expressed EGFP marker, shown in Figure 1.



Fig. 1A. LNCaP spheroids cultured on soft agar.

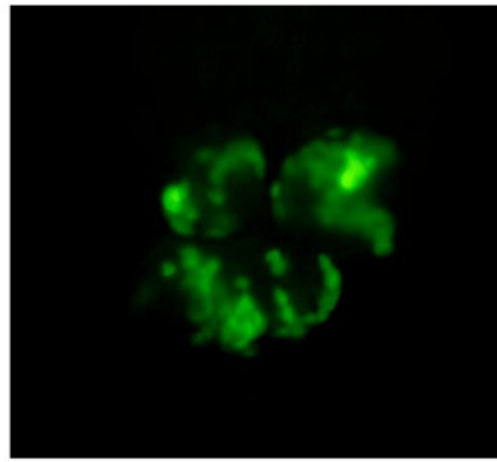


Fig. 1B. Expression of EGFP from same spheroids.

Normal human prostate stromal cells, PrSCs (purchased from Lonza), were cultured on glass coverslips or multi-chamber culture slides pre-coated with collagen I gel as proposed. PrSCs were seeded and cultured (arrows) in defined media (DMEM plus 0.5% BSA). Individual LNCaP spheroids of 150 mm diameter were pulled from the agar plates using 200 μ l pipet tips, mixed with 100 μ l of collagen type I and this was overlaid onto the existing PrSCs/collagen gels to produce the sandwich co-culture in defined DMEM/0.5% BSA media as proposed. Five-day co-cultures constructed with LNCaP spheroids (asterisk) producing HA-TGF- β 1, exhibit larger and more spread out spheroids as compared with control co-cultures as shown in Figure 2. In addition, cells from LNCaP spheroids expressing HA-TGF- β 1 appear to migrate more into stromal co-cultures as seen in Figure 2B. Co-culture experiments have been repeated twice with similar results, although these data are preliminary and have not yet been quantitated. Moreover, we have not yet evaluated the differentiation status of the stromal cells. These data do show,

however; that we can conduct the co-culture experiments and use fully defined media conditions.

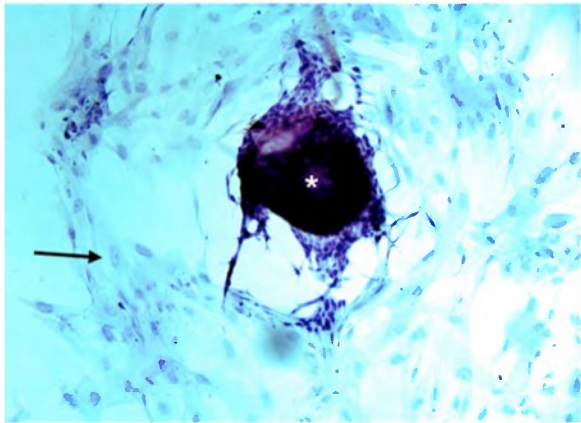


Fig. 2A. Control co-cultures.

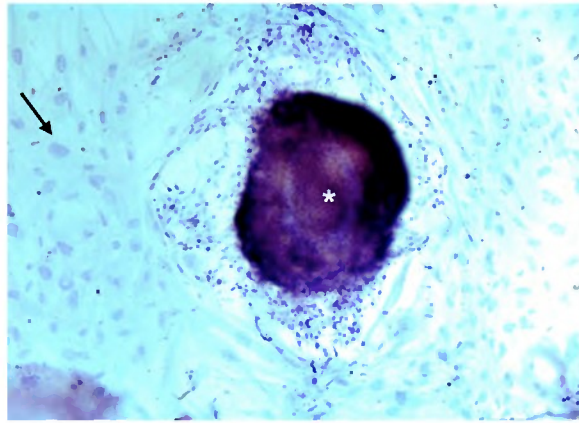


Fig. 2B. Co-cultures - LNCaP expressing HA-TGF-b1.

2. Multispectral imaging of human cancer tissue arrays

Our recent studies show that CD34+ cells co-expressing vimentin are observed differentially in human prostate cancer reactive stroma. We have used several different human cancer tissue arrays IHC stained for vimentin, CD34, CD11b, CD14, CD68, CD31, tenascin-C, and procollagen I. TMAs consisted of 60 prostate cancer patients, mammary, colon, lung, pancreatic, brain, and thyroid, and mixed tumor. The alkaline phosphatase fast red and HRP brown dual color with multispectral image analysis was used to evaluate and assign differential color and overlap as shown in Figure 3 (CD34 = brown and vimentin = red). Heterogeneous foci of vimentin+/CD34+ reactive stroma was observed in nearly all of the TMAs with the exception of brain and some of the mixed tumor samples that did not have interpretable data. In particular, prostate and pancreatic cancer exhibited the most extensive foci of what we have termed CD34+RS. See Figure 3 as an example of prostate gland staining. The most power aspect of this imaging system is the ability to obtain quantitative data on staining based on pixel density (Figure 4). This highly reliable method overcomes some of the limitations previously observed with standard immunohistochemistry by generating measurements that can be analyzed statistically.

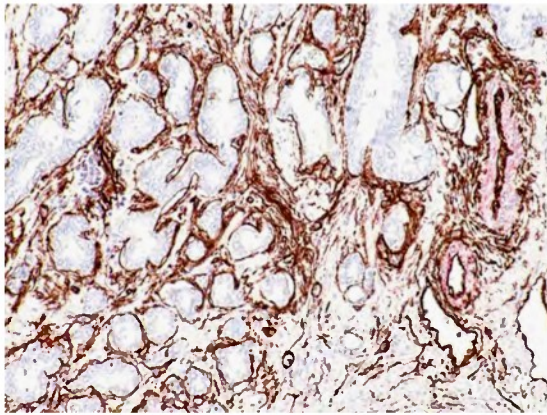


Figure 3A: Standard microscopy of dual label IHC method. Prostate cancer tissue with CD34+RS.

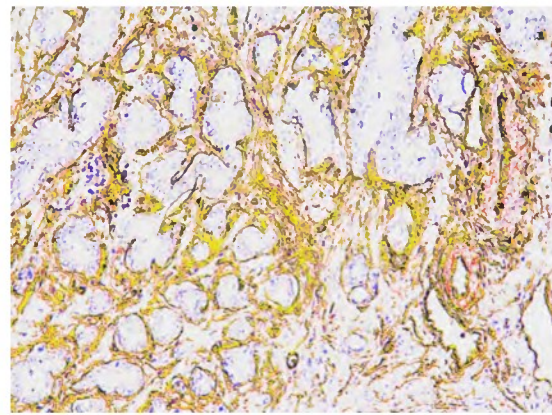
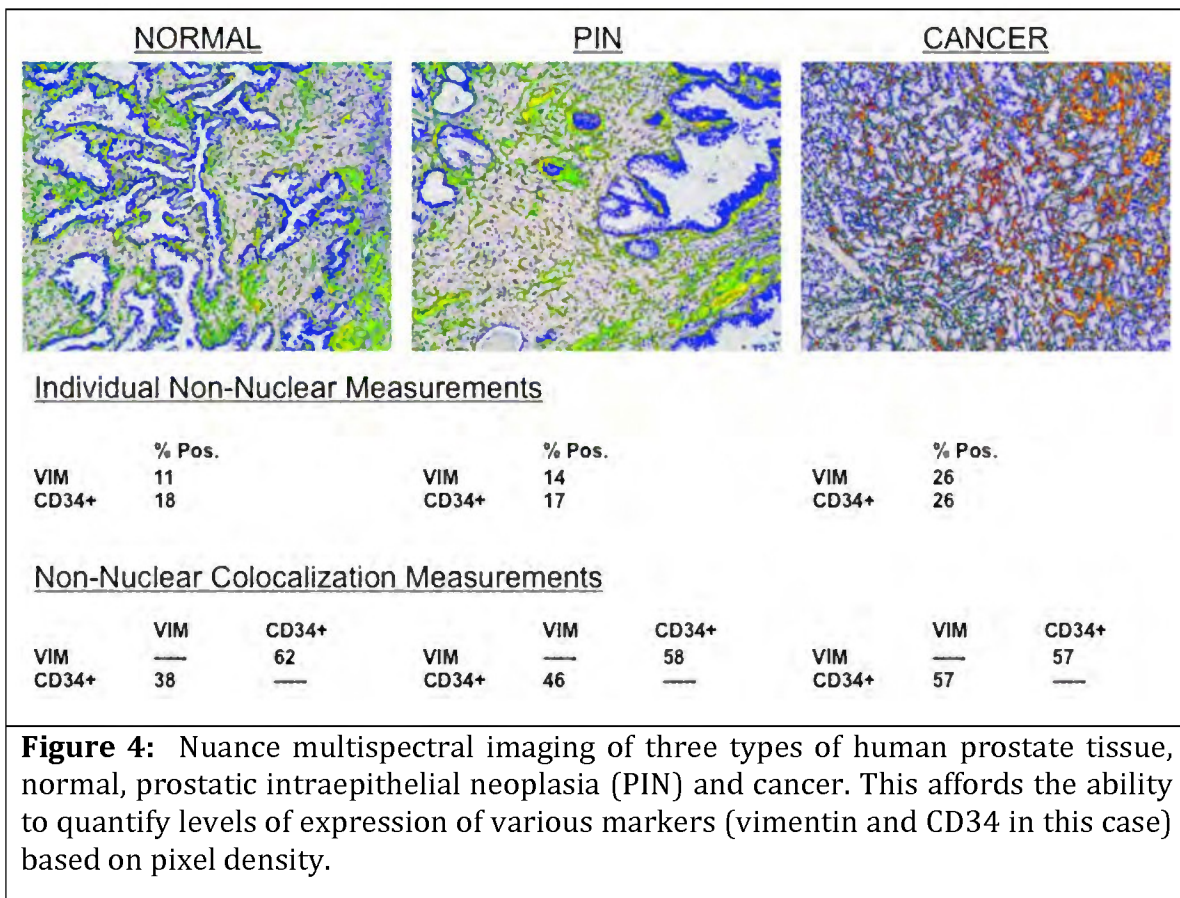


Figure 3B: Multispectral imaging shows cells co-expressing CD34 and vimentin in yellow. Vimentin = red, CD34 = brown



3. Adoptive transfer of CD34+ cells

We have used adoptive transfer of CD34+ and CD14+ human cells purified from human peripheral blood injected into the tail vein of nude mice that bear DRS LNCaP xenograft tumors. Preliminary analysis of these tumors show recruitment of cells that stain positive for human CD34 (see Figure 5). These cells assume a stromal cell-like phenotype typical of reactive stromal cells. We have not yet fully evaluated the tissues derived from the CD14+ adoptive transfer experiments. Although still preliminary, these studies suggest that we will see recruitment of circulating progenitors to reactive stroma. The next step is to address whether these cells differentiate to myofibroblasts in reactive stroma.

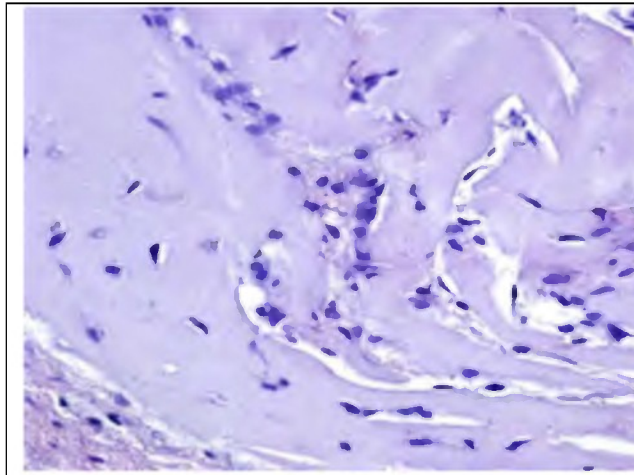


Figure 5A: CD34+ cells isolated from human peripheral blood were injected into nude mice containing human LNCaP prostate cancer xenografts. Preliminary data shows recruitment of stromal cells (panel A, H & E).

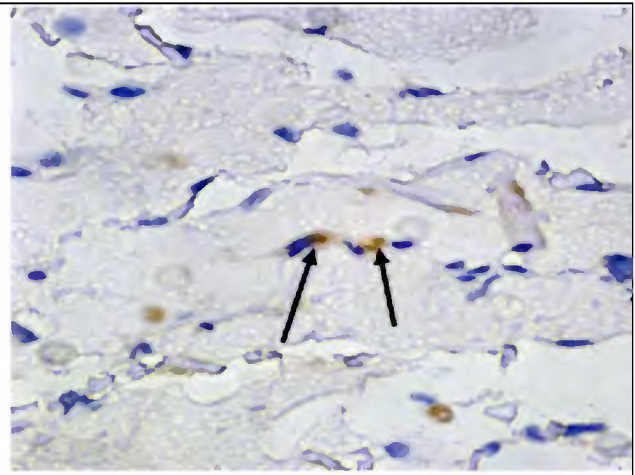


Figure 5B: A subset of cells were positive for human CD34 (arrows)